BINDING OF OESTRADIOL TO RECEPTOR-SUBSTANCES PRESENT IN EXTRACTS FROM CALF UTERUS

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1. Introduction

In the past few years considerable work has been accumulated on the binding of oestradiol(E) to both tissues and extracts from target organs like the uterus [1]. In this paper we wish to study in some detail the mechanism of oestradiol binding to some "receptor proteins", partially purified from calf uterus. It is shown by the use of several different technics of binding that two categories of oestradiol binding sites are involved. Binding of oestradiol to the first class of binding sites occurs at low oestradiol concentrations (around 10^{-9} M), and is slowly reversible (half life about 20 days at 0°C) (sites A); binding to the other class of sites occurs at much higher concentrations of oestradiol and is rapidly reversible (half life < 2 min) (sites B). We interpret the *in vivo* phenomenon of "retention" observed by Jensen and co-workers [1] as due to binding to the sites A, the phenomenon of "uptake" being caused by the binding to the sites B.

2. Methods

All experiments were carried out between 0–5°C, in a 0.04 M Tris buffer, pH 7.4, containing 0.0015 M EDTA, 0.002 M 2-mercaptoethanol, and 0.05 M KCl. Oestradiol 6.7 H_3, 30 c/m M was from Amersham. Radioactivity was measured in Bray's solution. Protein was assayed by the biuret method. Equilibrium dialysis was carried out according to Myer and Schellman [2]: 0.3 ml extracts, containing 1 mg protein/ml were equilibrated for 24 hr against 0.3 ml buffer containing different amounts of tritiated oestradiol (EH_3). To measure binding exclusively to the sites A, extracts (1 mg protein/ml) were labelled overnight with different amounts of EH_3. 0.1–0.3 ml aliquots were then either filtered through 4–12 ml columns of Sephadex G 25 coarse, or dialysed against large volumes of buffer for 18 hr. The amount of radioactivity in the void volumes, or in the dialysis bags was measured. The dialysis method was used to measure "specific activity" of the sites A: Moles of E bound/g protein/liter, when an extract, containing 1 mg protein/ml was kept overnight in the presence of practically saturating concentration, 2×10^{-8} M, of EH_3. Preparation of extracts. The buffer used was the same as described above, but KCl was omitted. 1 g scraped calf endometrial tissue was homogenised in 2 ml buffer in a glass-teflon Potter homogeniser, or 1 g total calf uterus in 2 ml buffer in the Waring-blendor. Homogenates were centrifuged at 100,000 × *g* for 90 min, pellets were discarded. (Supernatant = crude extract). Partial purification. If a crude extract is labelled with EH_3, the radioactive receptor-oestradiol complex sediments as a "8 S peak" on sucrose gradient or is eluted practically with the void volume when filtered on a Sephadex G 200 column [3,4]. We have shown that the uncomplexed, free receptor behaves similarly [5]. Accordingly, crude unlabelled endometrial extracts were fractionated by both methods, extracts from total uterus only by the Sephadex method. 1 ml extract was layered on a 8 ml 5–20% sucrose gradient and centrifuged for $3\frac{1}{2}$ hr at 60,000 RPM in the angle head of an MSE preparative ultracentrifuge. Fractions, corresponding to the "8 S peak" were pooled; or 20 ml extract was filtered through a 600 ml Sephadex G 200 column, and frac-

Table 1
Extraction and partial purification of E-receptor from calf uterus.

Origine of tissue (1 g wet weight)	Crude extract***			Partial purification (values given for 1 ml crude extract)					
				by ultracentrifugation			by Sephadex filtration		
	Volume ml	Protein mg/ml	Spec. act.*	Volume of pooled fractions	Protein mg/ml	Spec. act.*	Volume of pooled fractions	Protein mg/ml	Spec. act.*
Total uterus	2	15	2×10^{-10}	–	–	–	2.7	1	10^{-9}
Endometrium**	2	16	10^{-9}	3	1	4.6×10^{-9}	2.9	1	5.1×10^{-9}

* Specific activity: moles of E bound to sites A × protein g^{-1} × $liter^{-1}$ at saturation, as described under Methods.
** 1 g total uterus yields 0.1 g endometrial tissue.
*** About 30% of the E-receptor is extracted under the conditions of the experiment [1,12,13].

tions corresponding to the void volume were pooled. Quantitative data are summarised in table 1.

3. Results

3.1. *The two classes of E binding sites*

Equilibrium dialysis experiments were carried out first with endometrial extracts, which are much richer in receptor substances than the myometrium ([1,6] see also table 1). Extracts partially purified by ultracentrifugation or Sephadex filtration – as described in Methods – yielded identical results; routinely extracts from total uterus were thus partially purified only by Sephadex filtration. Fig. 1 shows the data obtained by equilibrium dialysis with endometrial extracts, and plotted according to Scatchard [7]. Quantitative data of these experiments, and of those with extracts from total uteri (not shown in fig. 1) are summarised in table 2. Fig. 1 shows, that at low E concentrations the Scatchard plot is apparently linear, at high E concentrations it becomes practically horizontal, indicating the presence of two receptor substances, one having a limited number of sites, and very high affinity (sites A, $K_A = 6.7 \times 10^9\ M^{-1}$), the other every high number of sites but low affinity (sites B). For the latter we found that the amount of E bound is proportional to the free E concentration up to 2×10^{-5} M, the limit of solubility of E, under the conditions of the experiment. Baulieu et al. [8] experiments on porcine endometrial extracts yield similar results. Extracts prepared from *total* uteri exhibit in all respect similar behaviour, the only difference being, that the number of the sites A is 5 × smaller (table 2). The finding, that the amount of sites A is smaller, but that of the sites B is apparently identical to the amounts found in endometrial extracts, is compatible with the results of Alberga and Baulieu [6] on rat uterus *in vivo*.

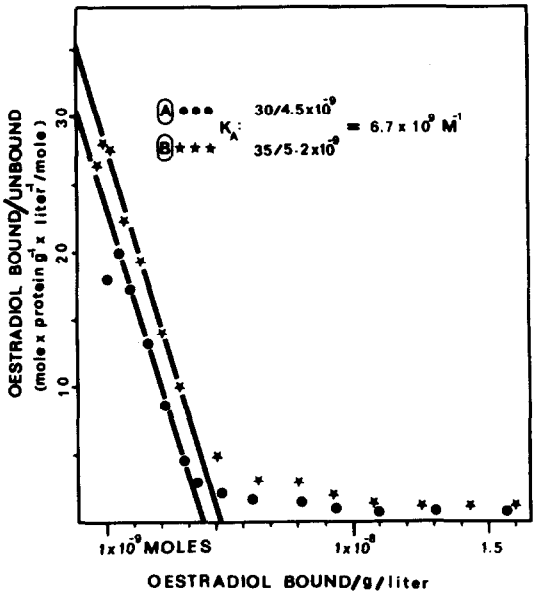

Fig. 1. Scatchard plot of equilibrium dialysis experiments, carried out with endometrial extracts partially purified by ultracentrifugation: A, and by Sephadex filtration: B.

Table 2
Binding constants of E-receptor substances.

Origine of extract	Method of partial purification	Experimental values: moles E/g/liter, as in figures						
		Equilibrium dialysis experiments				Experiments measuring binding of E only to the sites A	Theoretical values calculated for 1000 g tissue/liter**	
		Binding to the sites A			Binding to the sites B*		sites A	sites B
		nK (app.)	n (app.) moles	K_A (app.) M^{-1}	Ratio E bound/ unbound	Moles E bound at saturation	Moles E bound at saturation	Ratio E B/U
Endometrium	u.c.	30	4.5×10^{-9}	6.7×10^{9}	0.8	–		
Endometrium	Seph.	35	5.2×10^{-9}	6.7×10^{9}	1	5×10^{-9}	1.6×10^{-7}	33
Total uterus	Seph.	7	10^{-9}	7×10^{9}	0.82	10^{-9}	3×10^{-8}	25

* The ratio E B/U does not change up to 2×10^{-5} M.

** Examples: Sites A. 30% of the R is extracted when 1 g WW total uterus is homogenised in 2 ml buffer yielding a crude extract containing 15 mg protein/ml. The spec. act. is 2×10^{-10}, i.e. 2×10^{-13} moles of E are bound/mg protein/ml. Hence 1000 g WW total uterine tissue/liter would bind $1000 \times 3.3 \times 3 \times 15 \times 2 \times 10^{-13} = 3 \times 10^{-8}$ moles of E at saturation. Sites B: The same crude extract was purified 5 × by the Sephadex method, as judged from spec. act. of the sites A. The ratio E B/U is 0.82. Assuming that the R corresponding to sites B is extracted and purified in the same proportion as that corresponding to sites A the ratio E B/U for 1000 g WW total uterine tissue/liter would be $3.3 \times 3 \times 3 \times 0.82 = 24.6$. (See also table 1.)

3.2. *The reversibility of E binding*

In order to test the stability of the complex E-R we compared the binding data obtained by equilibrium dialysis with those obtained by two different technics either filtration on a Sephadex column equilibrated with a buffer which does not contain the hormone, or extensive dialysis against the same type of buffer (see Methods); both technics give quantitatively the same results as shown in fig. 2, an important fraction of E remains associated with some macromolecular components of the extract after both filtration and extensive dialysis. If we compare these results with those obtained by equilibrium dialysis, we see that the two set of data cannot be superimposed. When the amount of E "irreversibly" bound is plotted against the total E added, the points seem to follow a simple hyperbolic law, there is a tendency for saturation. Since the apparent binding constant measured in these conditions is almost the same as the one corresponding to sites A in the equilibrium dialysis experiment we conclude that the binding of E to the sites A is slowly reversible, or irreversible in the time course of our experiments. Since the E bound to the sites B, in the equilibrium dialysis experiment, is not preserved in the Sephadex or extensive dialysis ones, it is concluded that the complex E, sites B is rapidly reversible. As the results of the Sephadex filtration experiments, accomplished in 2 min, are identical with those of extensive dialysis for 18 hr, one can assume that the half life of the complex E, sites B is shorter than 2 min. We found in previous experiments [11] using endometrial extracts that the half life of the sites A is about 20 days at 0°C. Repeating this experiment with a partially purified extract from total calf uterus by the means of the extensive dialysis technics, we found a half-life of 20 days as well.

The challenging question whether the sites A and sites B are carried by the same molecule, resembling somehow the finding of Changeux et al. [9] concerning binding of acetylcholin to acetylcholinesterase, or whether they are on different molecular species, remains to be answered.

Jensen and co-workers [1] have shown that if E is injected to immature female rats, the hormone is

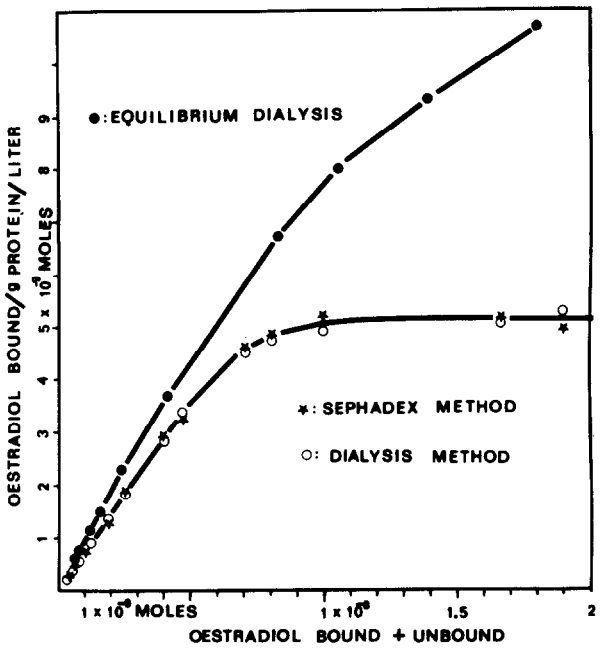

Fig. 2. Binding of E to the sites A, measured by the Sephadex method: * and by the extensive dialysis method: ○. For the sake of comparison the data of the equilibrium dialysis experiment (fig. 1) are plotted as well: ●.

rapidly accumulated in the uterus. A part of the accumulated E is rapidly eliminated, another part retained for several hours. The amount of E "taken up" is proportional at least to 1 μg E injected per animal, while the amount "retained" is only up to 0.1 μg. We suggest that the rapid, unsaturable and rapidly reversible "uptake" phenomenon is due to the binding of E to sites B, and the saturable, slowly reversible "retention" to the binding of E to sites A. Though our experiments were carried out with calf uterus, and Jensen's *in vivo* experiments with rats, we think that the basic phenomena of uptake, and retention must be the same in both cases. As Toft et al. [10] measuring binding to the sites A in extracts from total rat uterus by ultracentrifugation on sucrose gradients, found values identical with ours, we think that the comparison of quantitative data of our experiments with those of Jensen, is justified: 15 min after the injection of 0.5 μg E/animal, 10^{-7} moles of E are "taken up" by 1000 g uterus/liter. Our experiments show (table 2), that the sites B exhibit a ratio E bound/E unbound = 25, consequently if the sites B are responsible for "uptake", about 4×10^{-9} M unbound E should be found in the plasma, a value compatible with the *in vivo* experiment [1]. 2 hr after the injection of E, 2×10^{-8} moles of E are "retained" *in vivo*, and 3×10^{-8} moles bound to the sites A in our experiments, at saturation. As E is rapidly metabolised in the organism except in the uterus [1] we suggest tentatively that the physiological role of the sites B is to concentrate rapidly as much E as possible near to the sites A, which in turn "deprive" the sites B from E, and owing to their extreme stability retain the hormone for a considerable time.

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